IN VITRO DEVELOPMENT OF ANCYLOSTOMA TUBAEFORME, ANCYLOSTOMA CANINUM AND TOXOCARA CATI

by

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B.A., Rockhurst College, 1963
B.S., Kansas State University, 1967
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A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Parasitology
Department of Infectious Diseases

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1969

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INTRODUCTION

The purpose of this problem was the *in vitro* cultivation of the various stages of *Ancylostoma tubaeforme* Zeder, 1800; *Ancylostoma caninum* Ercolani, 1859; and *Toxocara cati* (Schrank, 1788) Brumpt, 1927 (syn. *T. mystax*) (Zeder, 1800).

Ancylostoma tubaeforme is the hookworm of the cat while *A. caninum* is the hookworm of the dog. They belong to the Phylum Nemathelminthes, Class Nematoda, Order Strongyloidea and Family Ancylostomatidae.

Toxocara cati is the roundworm of the cat. It belongs to the same phylum and class as *Ancylostoma* but is included in the Order Ascaroidea, Family Ascaridae.

This study was undertaken because these parasites are important in Veterinary Medicine and the development of an *in vitro* cultivation technique will provide a means of studying these parasites without the complicating factors of the host.

REVIEW OF THE LITERATURE

Although no research has been reported on *in vitro* cultivation of *Ancylostoma tubaeforme* or *Toxocara cati*, partially successful attempts to grow other hookworms and ascarids through various segments of the life-cycle have been reported.

In a single experiment, Weinstein (1954) reported development of eggs of *Necator americanus* to the filariform stage (67% yield) in 50% chick embryo extract (CEE); these were normal morphologically, but small in size. When CEE was supplemented with formaldehyde killed *Escherichia coli*, 98% developed to the filariform stage and were normal in size. Fifty per cent CEE, when diluted with beef heart infusion broth (1 part CEE:2 parts broth), resulted in higher yields of filariform larvae than CEE alone. Even when diluted 1:8, a yield of 59% filariform larvae was obtained. Broth alone would not support growth.

Using the same medium in which *Nippostrongylus muris* was successfully grown, Weinstein and Jones (1959) inoculated cultures with third-stage larvae of *Necator americanus*. Several worms developed a provisional buccal capsule, and a few reached the fourth stage. One fourth-stage female reached 1.7 mm in length and showed early differentiation of the genital tract.

McCoy (1930) grew *Ancylostoma caninum* eggs to infective stage on ordinary bacteriological agar inoculated with *Bacillus coli* (syn. *Escherichia coli*). The worms attained most rapid development at 37 C, and reached infective stage in 48 hours.

Weinstein (1949, 1953) reported growing *Ancylostoma caninum* from the egg to the filariform larva stage in the absence of living bacteria

by using either fresh chick embryo or rat-liver extracts containing penicillin and streptomycin. Larvae obtained from such a rat-liver culture gave rise to a normal infection in a puppy.

Komiya, et al., (1956) studied conditions under which Ancylostoma caninum adults could be maintained in vitro. They found that among the various physiological saline solutions, Krebs-Ringer's bicarbonate solution was most efficient for the survival of A. caninum; but addition of glucose to the solution resulted in a marked increase in survival time. Survival time in whole serum did not differ significantly with dilution to 50 or 75% with Krebs-Ringer's solution. In dog serum at 37 C the longest survival time was 6 weeks for male and 12 weeks for female worms. Addition of blood cells to the serum had no effect on survival time. Copulation in vitro took place in dog serum at 37 C and fertilized eggs were found until day 54.

Leland (unpublished data) advanced third-stage larvae of Ancylostoma caninum to the fourth-stage in a modification of Ae medium.

Sawada, et al., (1954) studied the development of Ancylostoma caninum larvae in the chicken embryo and found that a few third-stage infective larvae would form a provisional buccal capsule in 10 or 16 days.

Lawrence (1948) cultivated A. braziliense from the egg to the filariform (third) larval stage in a medium of fresh rabbit tissue and water agar with the addition of liver extract and ground, killed yeast.

Weinstein (1949, 1953) reported growing Ancylostoma duodenale from the egg to the filariform larval stage in the absence of living bacteria by using either fresh chick embryo or rat-liver extracts

containing penicillin and streptomycin.

Sawada, et al., (1954) inoculated chick embryos with third-stage A. duodenale larvae. They advanced some to the stage where sex could be distinguished and a buccal capsule was present.

Yasuraoka, et al., (1960) maintained adult A. duodenale in vitro for more than 4 months in human serum to which penicillin G potassium and dihydrostreptomycin was added.

Ackert, et al., (1938) succeeded in increasing the length of Ascaridia galli larvae by 53.8% in: 1.) incubating hen's eggs; 2.) starch-dextrose solution on a basic medium of cornmeal agar covered with sterile saline solution; and 3.) salt-dextrose solution on the same basic medium. No growth occurred in saline.

Guevara, et al., (1963) maintained adult Ascaridia galli in saline.

Various methods for obtaining large numbers of viable Ascaris lumbricoides var. suum larvae for in vitro cultivation work have been successfully tried (Pitts, 1948; Rogers 1958; Fairbairn, 1961; Cleeland and Lawrence, 1962).

Sofar, attempts to grow Ascaris lumbricoides var. suum in vitro through its complete life-cycle have been unsuccessful. Hoeppli, et al., (1938) kept the larvae of Ascaris alive in a medium containing the serum of a horse or guinea-pig, but did not observe development. Pitts (1962) advanced Ascaris larvae to 96.5μ in length and 59μ in diameter in Eagle's minimum essential medium supplemented with fresh rabbit serum. Forty-two days at 35-36 C were required for this development.

Cleeland (1963) using either Eagle's medium or medium-199 with 20% calf or bovine serum advanced Ascaris in length to 2,300μ by 83μ in diameter; but it took 100 days and specific conditions which

included an initial larval concentration of 20,000 or more per 10 ml of medium, 37.5 C and partial anaerobiasis.

Levine and Silverman (1969) advanced Ascaris lumbricoides var. suum second-stage larvae to the third-stage in two media. The best medium as judged by development rate was Caenorhabditis briggsae medium supplemented with 16.7% pig serum. The highest survival rate was obtained in Eagle's minimum essential medium constituted in Earle's salt solution plus 16.6 to 22.2% pig serum.

MATERIALS AND METHODS

Source of Organisms

Two cats, a young adult female (Cat-1) and a 3 month old male kitten (Cat-2), naturally infected, were the source of Ancylostoma tubaeforme. Five hundred third-stage infective larvae of both Cats 1 and 2, obtained from Baermanized 7-day old vermiculite-fecal cultures, were used to inoculate the subsequent donor cat (Cat-3). Larvae were suspended in tap water and injected subcutaneously in the shoulder region.

The third-stage infective larvae of Cat-3 were used for subsequent injections in Cat-4 (1,000 larvae) and Cat-5 (4,200 larvae).

A naturally infected dog which died of an overdose of barbiturate anesthetic provided the Ancylostoma caninum adults.

The adult Toxocara cati were obtained from Cat-2 at necropsy.

Identification of all adult worms were made following termination of the particular experiment in which they were used. Ancylostoma tubaeforme and A. caninum were differentiated according to Burrows (1962). Toxocara cati were identified according to Monnig (1962).

Recovery and Preparation of Eggs

Eggs were recovered from feces by the gradient technique of Marquardt (1961) which was modified to include three to six sedimentations of the feces instead of only one as described by Marquardt.

The eggs obtained were washed four times in cold tap water, subjected to White's solution (prepared according to Weinstein, 1953) for 24 minutes including 5 minutes for centrifugation. They were then rewashed in either sterile distilled water or Balanced Salt Solution-

Antibiotics (BSSA), (prepared according to Leland, 1963). The eggs were resuspended in a quantity of sterile distilled water or BSSA, sufficient to approximate the desired number of eggs per 0.2 ml.

Eggs adhering to the tube walls were processed in the same manner in the tube to which they were attached.

Preparation of Third-stage Larvae

The eggs used to produce larvae came from Cats 1, 2 and 3. The third-stage infective larvae were obtained from 7-day vermiculite-feces cultures. The larvae were harvested from the vermiculite-feces cultures with the Baerman apparatus.

The harvested larvae were treated in the following manner: They were washed 3 to 5 times in tap water, once in sterile demineralized water, artifically exsheathed in Clorox® solution (0.2 ml Clorox in 10 ml sterile demineralized water) and washed 3 to 5 times in sterile demineralized water. The larvae were then allowed to stand one-half hour in BSSA and/or Polymyxin B in sterile demineralized water solution (0.1 mg/ml). The larvae were resuspended in sufficient BSSA to obtain the desired number of larvae per standard inoculum.

The approximate number of larvae per 0.2 ml was determined from counts of 0.2 ml aliquots of larval suspension.

Bacteriologic tests to detect aerobes included inoculation of 1 ml of medium (on termination of the culture) into Trypticase Soy Broth. After 48 hours Salmonella-Shigella (S-S) and blood agar plates were inoculated from the broth.

Tests to identify the bacterial contaminant in some cultures

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* Clorox (sodium hypochlorite, 5.25% by weight), Clorox Chemical Company, Oakland, California.

included inoculation of blood agar, Salmonella-Shigella and McConkey's agar. The antibiotic sensitivity of organisms grown on blood agar was also tested.

Preparation of Adult Worms

Adult Ancylostoma tubaeforme were obtained at necropsy from Cats-1, 2 and 3. A. caninum adults were obtained from a dog which died of barbiturate overdose. Adult Toxocara cati came from Cat-2.

Worms were retrieved and prepared for inoculation in the following manner: the intestinal contents and mucosal scrapings were washed with tap water through a No. 50 sieve, washed 4 or 5 times in BSSA at 38 C and incubated 20 minutes in BSSA at 38 C prior to inoculation into media.

Each culture system contained 1 to 10 females and 3 to 7 males.

Media

The media used in these experiments were Ae (Leland, 1963) and modifications of Ae medium. Modification of Ae was characterized by omission or alteration of the serum component.

Ae medium was prepared as reported by Leland (1963). Composition of the medium per 100 ml was:

50 ml	chick-embryo extract (CEE_{50})
15 ml	serum from helminth-free calves
15 ml	sodium caseinate
5 ml	vitamin mixture
5 ml	liver extract
1 ml	antibiotics (penicillin, streptomycin and mycostatin)
9 ml	Balanced Salt Solution-Antibiotic (BSSA)
100 ml	

The pH was adjusted to 7.2-7.3 with sterile sodium bicarbonate or carbon dioxide gas.

The 11-day old chick embryos were removed aseptically from eggs and washed in ice-cold BSSA. One ml of BSSA was added for each 1 gm of embryos and this was blended for 3 minutes in a blender. This mixture was allowed to set 1 hour in the blender jar at 4 C. The mixture was divided equally in screw-cap centrifuge tubes and centrifuged 20 minutes at 4 C and 1,800 G. After centrifugation it was allowed to settle 20 minutes in the centrifuge at 4 C, drawn off and pooled. All CEE_{50} was made the same day it was incorporated in medium.

Serum used was obtained from the blood of immature animals. The blood was allowed to clot one hour at room temperature and placed under refrigeration overnight at 4 C. The serum was centrifuged one hour at 4 C and 1,800 G, removed and stored in the frozen state at -20 C until used.

The sodium caseinate was prepared by the addition of 4 gm vitamin-free casein (Nutritional Biochemical Inc.) and 50 mg cystine to 150 ml distilled water. 1N sodium hydroxide was added until the solution was complete (pH 11). The solution was then back-titrated to pH 7.2 using 1N hydrochloric acid, and the volume adjusted to 200 ml with distilled water. It was sterilized by autoclaving at 15 lbs pressure for 15 minutes and stored frozen at -10 C until used.

The vitamin mixture was Basal Medium Eagle (100X concentrated) formulated by Microbiological Associates Incorporated. The undiluted commercial mixture contained 0.1 mg/ml of biotin, choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, thiamine and 0.01 mg/ml of riboflavin.

The liver concentrate was prepared by addition of Sigma Liver Concentrate to BSSA at a proportion of 1 gm liver concentrate to 50 ml BSSA. This mixture was filtered through a Millipore Stainless Filter at a pressure of 10-13 inches of mercury with Prefilter AP 200 4200 42 mm and MP filter HA (0.45 microns) 47 mm HAWP04700. The filtrate was then stored in the frozen state at -10 C until used.

The antibiotic mixture used in all media and in BSSA was made as follows: to 50 ml sterile water was added 2 gm of Squibb crystalline dihydrostreptomycin sulfate, 2 million units Squibb buffered potassium penicillin G and 500,000 Squibb Nystatin (mycostatin). This mixture was shaken well and stored in the frozen state at -10 C until used.

The Balanced Salt Solution-Antibiotic (BSSA) was formulated as follows: to 878 ml sterile distilled demineralized water was added 100 ml Earle's concentrate, 4 ml phenol red, 20 ml sterile sodium bicarbonate. The pH was adjusted to 7.2-7.3 with sterile carbon dioxide gas. Five ml antibiotic mixture (composition as previously described) was then added and the pH further adjusted to 7.2-7.3 with sterile sodium bicarbonate or sterile carbon dioxide gas as necessary. This was stored under refrigeration at 4 C until used.

Media were compounded the same day the CEE_{50} was prepared. Manipulation of CEE_{50} was carried out with the containers in crushed ice. Glassware was chilled before use and tubes, flasks and bottles were of the screw-cap type with non-toxic rubber liners within the caps.

Following combination of the components and adjustment of the pH to 7.2-7.3, the media were dispensed in 2 ml amounts to screw-cap culture tubes (16 X 125 mm) by means of a Salvarsan flask fitted with

an automatic syringe. The tubed media were quickly frozen in an alcohol-dry ice bath and stored at -20 C.

The following media were modifications of Ae medium where calf serum was replaced by 1.) feline serum not characterized according to the age of the donor, Af; 2.) feline serum of an adult cat, Afo: 3.) feline serum of sexually immature cats, Afy; 4.) swine serum, Ap; and 5.) an equal volume of BSSA, A-s.

Medium Ap199 was a modification of Ae medium where the 9 ml blank of BSSA (Leland, 1963) was replaced by 3.1 ml of 10X concentration of medium 199 and 5.9 ml sterile water; calf serum replaced by pig serum.

Media in which eggs were cultivated were: A-s, Afy, Ae, Ap and distilled water.

Third-stage larvae were cultivated in Af, Afo, Afy, Ae, Ap199 and BSSA as a control.

Adult worms were maintained in A-s and Afy.

Culture Manipulation

The eggs were incubated in a roller drum at 25 C for 10 to 11 days, transferred to fresh medium and incubated in a roller drum at 38.5 C for the remainder of the culture period. Transfer to new medium was made every fourth day for the first 5 to 6 times followed by weekly changes until termination.

Larval cultures were transferred to new medium every four days for the first 6 to 12 changes followed by weekly transfers until termination. The larval cultures were incubated at 38.5 C in a roller drum for the entire culture period.

Adult worms were maintained at 38.5 C in stationary culture bottles with 4 ml medium or in standard screw-cap culture tubes with 2 ml of

medium. The roller drum was used with the standard screw-cap culture tube. Medium was changed daily for the first 2 or 4 days followed by medium change every third day to termination of the culture.

RESULTS

Cultivation Initiated from the Egg

Data concerning egg cultivation is presented in Table 1.

Eggs hatched in all media. The percent hatch of eggs cultivated in medium without the serum component (A-s) was low. With the exception of Tube 8, the percent hatch of eggs cultivated in medium with serum was high.

Third-stage larvae developed in A-s, Afy, Ae, and Ap media. Third-stage larvae did not develop in distilled water (Tube 1); but did develop in distilled water in Tube 2 where much debris was observed. The percentage of larvae cultivated to third-stage was lower in medium without serum than in medium with serum.

Fourth-stage larvae were produced in the systems which employed: 1.) A-s medium at 25 C for 11 days followed by medium Afy at 38.5 C, and 2.) Afy medium at 25 C for 11 days followed by the same medium at 38.5 C; the percent advancement was higher in the former. The fourth-stage larvae produced *in vitro* appeared normal (Plate I , Fig. 1). Minimum development time was 31 days.

Cultivation Initiated from Third-Stage Larvae

Data relating to cultivation of third-stage larvae are presented in Table 2.

All media maintained the third-stage larvae. Larvae cultivated in Afy, Afo, Af and Ae media were active and appeared normal throughout the culture period. Ap 199 medium did not maintain larvae well. Many larvae died after developing numerous swellings on the cuticle and adhered together in masses. Some larvae did remain normal and active

Table 1. Development of Egg Cultures in Various Media.

Tube No.	1	2	3	4	5	6	7	8	9	10	11
Medium at 25 C	H_2O	H_2O	A-s	A-s	Afy	Afy	Afy	Afy	Ae	Ap	Ap
Medium at 38.5 C	-	Afy	Afy	Afy	Afy	Afy	Afy	Afy	Ae	Ap	Ap
Days in Culture at 25 C	10	10	11	11	10	11	11	11	10	10	11
Days in Culture at 38.5 C	-	10✓	127	109	82	72	72	109	82	82	72
No. of First and Second-stages	0	+**	100	360	18	112	21	46	7	9	74
No. of Third-stage	0	+**	2944	1020*	465	1727*	1917*	1449*	303	360	1407
No. of Fourth-stage	0	0	7	3	0	1	1	1	0	0	0
Total Number of Larvae	-	-	3051	1383	483	1840	1939	1496	310	369	1481
No. of Unhatched Eggs	-	-	5134	10100	11	82	32	5015	1	1	686
Percent Hatched	-	-	37.3	12.0	97.8	95.9	98.4	23.0	99.7	99.7	76
Percent Third-stage	-	-	35.8	8.8	94.1	89.8	97.2	94.8	97.4	97.2	64.9
Percent Fourth-stage Developed from Third-stage	-	-	0.2	0.3	-	0.1	0.1	0.1	-	-	-
Fourth-stage First Detected (days)	-	-	31	69	-	38	53	109	-	-	-
Average Length of Fourth-stage (mm)	-	-	1.42	1.25	-	2.13	1.33	1.54	-	-	-

* Dead Third-stage Larvae with Provisional Buccal Capsule Observed During Culture Period
** Observed but Not Counted
✓ Terminated Due to Contamination

during the entire culture period.

Third-stage larvae were advanced to fourth-stage in Af, Afo, Afy and Ae. No fourth-stage larvae were present in medium Ap 199. One third-stage larvae in each of Tubes 22 and 23 developed a provisional buccal capsule (late third stage, Matsusaki, 1950) about 29 days in culture and both died within 4 days. Dead third-stage larvae with provisional buccal capsules were also observed in Tubes 4, 5, 11 and 12.

The percentage fourth-stage larvae ranged from 0 to 0.5%. There was no difference in percent larval advancement between the media which contained serum from sexually mature, immature, or both, cats.

Eight fourth-stage larvae died in the process of ecdysis to the adult stage (Plate II, Fig. 3).

Minimum development time to fourth-stage was 7 days. In Tube 3 one fourth-stage larva developed at 78 days in culture. This culture was maintained in Af medium to 71 days at which time Afy medium was substituted. Fourth-stage larvae were first detected at 7 days in Afy, 17 days in Afo and 14 days in Ae.

Average lengths of *in vitro* fourth-stage larvae ranged from 1.19 to 2.26 mm which falls within normal range for *in vivo* larvae (Matsusaki, 1950).

Life-span of fourth-stage larvae ranged from 5 to 14 days with an average of 9 days. At no time were more than 3 live fourth-stage larvae observed per culture.

Studies of the bacterial contaminant showed it was *Escherichia coli* and sensitive to Polymyxin B. The use of Polymyxin B (0.1 mg/ml) in the pre-inoculation procedure eliminated detectable contamination. Tests to determine the presence of aerobic bacteria were negative.

Table 2. Development of Third-stage Larvae in Various Media.

Tube No.	Medium	Days in Culture	No. of Third-stage	No. of Fourth-stage	Total No. of Worms	Day Per cent Fourth-stage First Detected	Av. Length Fourth-stage (mm)
1	Af	113	206	0	206	-	-
2	Af	113	159	0	159	-	-
3	Af	107	775	1	776	0.1	78
4	Af	52	2919	3**	2921	0.1	21
5	Af	78	947	5**	952	0.5	16
6	Af	53	2107	1	2108	0.04	15
7	Af	53	1211	1	1212	0.1	26
8	Af	53	1627	0	1627	-	-
9	Afo	41	2907	3	2910	0.1	21
10	Afo	41	2729	3	2732	0.1	17
11	Afo	41	1727	2**	1739	0.1	17
12	Afo	41	1438*	3**	1441	0.2	17
13	Afy	41	4260	3	4263	0.01	17
14	Afy	41	2717	2	2719	0.1	32
15	Afy	41	3499	6	3505	0.2	21
16	Afy	41	3016	2	3018	0.1	7
17	Ae	107	515	0	515	-	-
18	Ae	78	535*	2	537	0.4	14
19	Ae	78	480	0	480	-	-
20	Ae	53	1233*	0	1233	-	-
21	Ae	53	921	1	922	0.001	38
22	Ap 199	78	430*	0	430	-	-
23	Ap 199	78	208*	0	208	-	-
24	BSSA Control	26	1450	-	1450	-	-
25	BSSA Control	21	775	-	775	-	-
26	BSSA Control	6	200	-	200	-	-
27	BSSA Control	23	1650	-	1650	-	-

* Dead Third-stage Larvae with Provisional Buccal Capsule Observed During Culture Period.

** Developed to Fourth-stage Larvae Undergoing Ecdysis to Adult-stage.

Maintenance of Adult Worms

Data relating to adult maintenance are found in Table 3.

Ancylostoma caninum adults were maintained a maximum of 62 days. The females produced eggs for 62 days and the eggs hatched to larvae for 34 days. Copulation was observed once in vitro. Males survived an average of 17 days in culture; maximum survival was 22 days. Females survived an average of 37 days and maximum survival was 62 days.

Worms in Tube 5 were maintained for the first 11 days in A-s medium then transferred to Afy medium with no change in egg laying or larval hatching capacity. Use of the stationary culture bottle after the fifth day in culture had no effect. Tubes 1, 2, 3 and 4 were discontinued due to contamination.

Ancylostoma tubaeforme adults were maintained a maximum of 49 days. The average survival time in culture for males was 20 days with a maximum of 41 days while the average for females was 30 days with a maximum of 49 days. The average and maximum figures for individual cultures appear in Table 3. Females produced eggs in culture 41 days and the eggs hatched to larval-stage for 25 days.

Tubes 6, 7, 8 and stationary culture bottles 10 and 12 were discontinued due to contamination.

Toxocara cati adults were maintained in Tube 13 for 13 days. During the entire culture period eggs were produced. Both the male and female died on day 13.

Table 3. Maintenance of Adult Worms.

	Ancylostoma caninum					Ancylostoma tubaeforme							Toxocara cati
Culture Number	1Δ	2Δ	3Δ	4Δ	5✓	6Δ	7Δ	8Δ	9✓	10✓	11✓	12✓	13Δ
Number of Worms male	3	3	3	2	4	3	4	3	7	7	7	7	1
Number of Worms female	3	4	4	4	8	1	1	1	10	10	10	10	1
Days in Culture	5*	5*	4*	4*	62	21*	21*	21*	49	21*	41	25*	13*
Days Egg Produced	5	5	4	4	62	21	18	21	41	21	25	25	13
Days Larvae Hatched	5	5	4	4	34	7	7	0	25	21	25	25	-
Av. Life in Culture, male (days)	5	5	4	4	17	11	21	16	25	12	18	13	13
Av. Life in Culture, female (days)	5	5	4	4	37	21	21	21	30	12	28	20	13
Max. Life in Culture, male (days)	5	5	4	4	22	13	21	21	41	18	41	25	13
Max. Life in Culture, female (days)	5	5	4	4	62	21	21	21	49	18	41	21	13
Copulation Observed	-	-	-	-	+	-	-	-	-	-	-	-	-

* discontinued due to contamination
✓ cultured in a stationary culture bottle
Δ cultured in standard culture tube

DISCUSSION

Cultivation Initiated from the Egg

Eggs cultivated in sterile distilled water (Tube 1) produced first-stage larvae which died shortly after hatching, probably due to lack of food. In Tube 2 eggs hatched and larvae developed to third-stage in distilled water probably because nutrients were present in the form of debris and bacteria.

Cultures initiated in media with serum had a higher percent hatch and percent larvae developing to third-stage than cultures initiated in media without serum. Serum apparently enhanced percent hatch and percent development to third-stage. Leland (1967) reported that serum may be inhibitory to the non-parasitic stages of Cooperia punctata. Serum inhibition apparently was not the case with the first and second stages of Ancylostoma tubaeforme.

There was little difference in the percent advancement to fourth-stage (Table 1, Tubes 3, 4, 5-11) when eggs were cultured in 1.) A-s medium at 25 C for 11 days followed by medium Afy at 38.5 C or 2.) Afy medium at 25 C for 11 days followed by 38.5 C.

The minimum development time from third to fourth-stage larvae was longer in vitro (31 days) than reported for larvae in vivo (4 days) by Okoshi and Murata (1967). The average length of fourth-stage larvae in vitro was within the normal range recorded for larvae in vivo by Matsusaki (1950).

The variations in the interval between transfers or method of egg preparation for inoculation into media had no discernable effect on percent hatch or development to fourth-stage.

Cultivation Initiated from Third-stage Larvae

There was no difference in the percentage of fourth-stage larvae produced when either calf or feline serum was used in the medium. Medium with immature feline serum reduced the development time of third to fourth-stage from 14 to 7 days. The value of serum from immature animals in nematode cultures (Leland, 1963) is further substantiated by these results. The discussion and conclusions on length of the fourth-stage larvae originating from egg cultures also applies to cultures originating from third-stage larvae. The number of fourth-stage larvae present at any one time was not great. The reason for this is conjectural but could be a form of inhibition where some limiting factor(s) such as larval excretory products or limited quantity of a nutrient prevent a greater percentage to advance to the next stage.

The percent of larvae advanced to fourth-stage was small. The fact that advancement occurred is significant for it provides a basis of comparison in future work to improve the percent advanced. The history of *in vitro* cultivation of *Cooperia punctata* (Leland, personal communication) showed an initial low percentage of advancement. Additional experimentation to determine optimal growth conditions resulted in greater percentage advancement.

Ecdysis of fourth-stage larvae to the adult-stage occurred in part but the larvae died before completion of the process (Plate II, Fig. 3). No explanation can be offered for the incomplete ecdysis particularly in view of the ease of adult-worm maintenance *in vitro*. This remains as the last portion of the life-cycle of *Ancylostoma tubaeforme* to be completed *in vitro*.

The slight variations in culture transfer schedule or method of

larval preparation for inoculation into media had no detectable effect on the percentage third-stage larvae advanced to fourth-stage.

Maintenance of Adult-worms

Ancylostoma caninum adults were maintained a maximum of 62 days in vitro. This was 20 days less than Komiya, et al., (1956); but no conclusions can be drawn since the age of the adults used in this experiment was unknown. Komiya, et al., (1956) used adults from recently patent artificial infections in their experiment. Eggs which hatched to larvae were produced for 34 days whereas Komiya, et al., (1956) observed no larvae after 28 days.

Afy provided a medium for limited in vitro maintenance of adult Ancylostoma tubaeforme. No copulation was observed in vitro and eggs laid in culture produced larvae for only the first 25 days.

The present technique and medium with some improvement and modification may be of use for investigation of adult-worm physiology, metabolism and anthelmintic studies in vitro for Ancylostoma caninum and A. tubaeforme.

ACKNOWLEDGMENTS

The author wishes to acknowledge advice and guidance given by Dr. Stanley E. Leland, jr. throughout the research program.

Thanks are due to Dr. Merle F. Hansen who stimulated the author's early interest in parasitology.

Special gratitude is extended to Dr. W. D. Lindquist for criticism of this text and help in expanding the author's knowledge of library research.

Mrs. Carol Sansevera, Mrs. Carol Church, Miss Pat Farrell and Mr. Dan Enterline are acknowledged for technical assistance.

The author is indebted to Dean C. E. Cornelius for granting Senior Special Studies Status to undertake this work.

The author owes a debt of gratitude to the Department of Infectious Diseases, the National Science Foundation and Chemagro Corporation for partial support of the research.

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APPENDIX

EXPLANATION OF PLATE I

Fig. 1 *Ancylostoma tubaeforme*. A fourth-stage larva produced *in vitro*. A third-stage larva is included to show the increase in size that occurred, X 60.

Fig. 2 *Ancylostoma tubaeforme*. Fourth-stage larva showing provisional buccal capsule, X 370.

PLATE I

Fig. 1

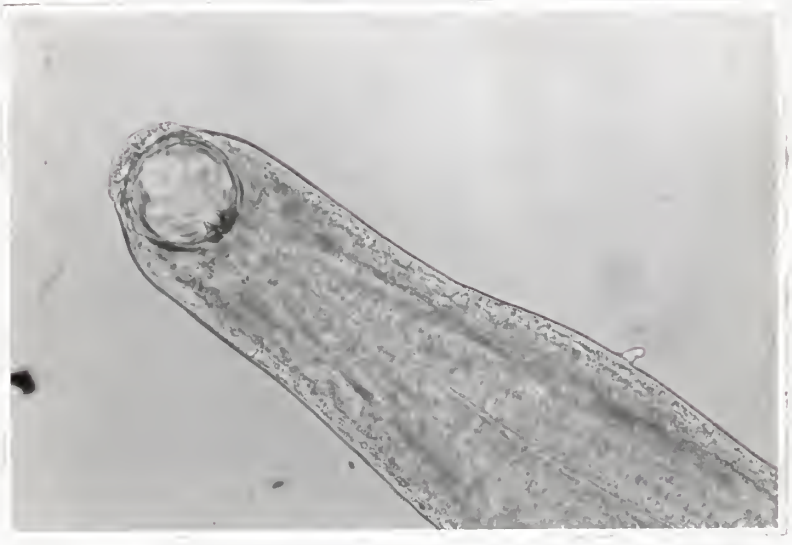

Fig. 2

EXPLANATION OF PLATE II

Fig. 3 *Ancylostoma tubaeforme*. Fourth-stage larva in fourth molt. Third-stage larvae included to show size increase *in vitro*, X 60

Fig. 4 *Ancylostoma tubaeforme*. Fourth-stage larva which died in process of ecdysis *in vitro*, X 370.

PLATE II

Fig. 3

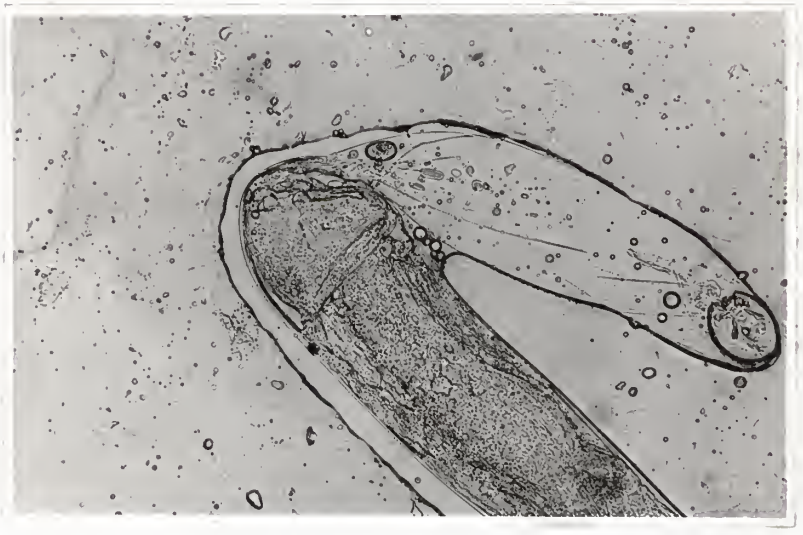

Fig. 4

IN VITRO DEVELOPMENT OF ANCYLOSTOMA TUBAEFORME, ANCYLOSTOMA CANINUM AND TOXOCARA CATI

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Ancylostoma tubaeforme was cultivated *in vitro* from the egg to advanced fourth-stage in a modification of Ae medium (Leland, J. Parasit. 49:600-611, 1963). Ae was modified by including cat serum in the medium in place of calf serum. Advancement from egg to third stage was completed in eleven days and from third to fourth stage in 31 days in the same system. When third stage larvae originating from vermiculite-fecal cultures were inoculated into the medium, advancement to fourth-stage was made in 7 days. However, the number of larvae reaching fourth-stage did not exceed 0.5%. Many of the fourth-stage larvae died in the process of ecdysis to fifth-stage. When the serum component of the medium was from immature rather than mature cats, advancement time from third to fourth-stage was reduced by half. Adult *Ancylostoma tubaeforme*, *A. caninum* and *Toxocara cati* from *in vivo* were also maintained in the modified Ae medium 49, 62 and 13 days, respectively. Copulation of *A. caninum* was observed *in vitro*.